Proline as a Factor in Distiller's Dried Solubles Which Stimulates Cellulose Digestion by Rumen Microorganisms

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Rumen stimulatory factors in corn distiller's dried solubles were completely extracted in water, precipitated with ethanol, and not destroyed by acid hydrolysis. The amino acids in a hydrolyzate of the ethanol precipitate from the water extract were separated into four groups by paper chromatography. Only the fraction containing proline gave significant increase in cellulose digestion, but was significantly less active than unchromatographed material. Proline was present in all active fractions but not in sufficient quantities to explain the response obtained. Determination of free proline and valeric acid, and cellulose digestion in tubes containing added proline or valeric acid after 6, 12, 18, and 24 hours of fermentation suggested that proline was converted to valeric acid during the 0 to 12 hours when no cellulolytic response was noted. During periods of significant cellulolytic response (12 to 24 hours), the results suggest that valeric acid was metabolized by the cellulolytic microorganisms. There was no indication that valeric acid was converted to proline.

Factors which enhance cellulose digestion by rumen microorganisms in vitro have been found in several protein-rich feedstuffs. Corn distiller's dried solubles (DDS) is recognized as a potent source of these unidentified factors, and previous work has revealed several chemical and physical properties of these factors (*7*), but has not definitely established their identity. Several organic compounds have been shown to stimulate cellulose digestion by rumen microorganisms in vitro, and some have been related to the stimulatory activity in natural feeds. Dehority *et al.* (*3*) indicated that the activity in yeast, casein, and alfalfa extract was related to some amino acids. Proline seemed to be responsible for a large part of the activity. Therefore, this work was initiated to study possible relationships between cellulolytic activity in DDS and its proline content.

Experimental and Results

In vitro cellulose digestion by washed cell suspensions of rumen microorganisms was used to assay different fractions of DDS for cellulolytic activity.

Ruminal ingesta were obtained 12 to 14 hours postprandial from a steer receiving a maintenance ration of alfalfa hay supplemented with corn, DDS, minerals, and vitamins A and D. Microorganisms were prepared and incubated as described by Cheng *et al.* (*2*). Triplicate 18-hour fermentations were conducted for each treatment. The means test of Tukey (*8*) was used for comparison of treatment means.

The DDS was a composite of five sources. The proximate analyses were: dry matter, 92.3%; crude protein, 26.5%; ether extract, 8.4%; ash, 8.9%. DDS was fractionated by various means to concentrate the active factors. A water extract of DDS was prepared by suspending a finely ground sample in 5 volumes of water, stirring for 30 minutes at 60° C., cooling to room temperature, straining through cheesecloth, and centrifuging at 20,000 G. The supernatant was collected and kept frozen until used as a treatment in the fermentation tubes or subjected to further fractionation. Little *et al.* (*7*) reported that 50 mg. of DDS per 20 ml. of in vitro fermentation medium resulted in maximum cellulose digestion. Unless otherwise stated, fractions equivalent to 50 mg. of DDS were used.

Responses of in vitro cellulose digestion to equivalent levels of DDS, water extract of DDS, and residue from water extraction are shown in Table I. The cellulolytic factors appeared to be completely extracted in water.

The water extract was fractionated further to concentrate the protein portion. It was freeze-dried with acetone and solid carbon dioxide at 5 mm. of Hg pressure, resuspended in 95% ethanol precooled to 3° C., refrigerated at 3° C. for 12 to 14 hours, centrifuged at 20,000 G, and resuspended in distilled water.

The results of adding this fraction to the fermentation tubes are shown in Table II. Most of the activity in the water extract appeared in the alcohol precipitate along with the proteins.

The alcohol precipitate was subjected to varying degrees of hydrolysis to determine stability of its cellulolytic activity to hydrolysis. The alcohol precipitate fraction was added to 6*N* HCl at a ratio of 1 mg. of protein to 5 ml. of acid and refluxed at 100° C. Samples taken every 2 hours in the first trial and after 10 and 24 hours in the second trial were added to the fermentation tubes after evaporating off the acid and redissolving in distilled water.

As shown in Table III, acid hydrolysis did not significantly affect the cellulolytic activity of the fraction precipitated by alcohol.

To determine possible relationships between the cellulolytic activity of DDS and proline, proline was isolated from a fraction of DDS by ascending paper chromatography. The amino acids in a hydrolyzate of the alcohol precipitate fraction were partitioned using Whatman's No. 1 filter paper and a 4:1:5 mixture of butanol, acetic acid, and water. After 24 hours, control strips were developed with ninhydrin spray.

Table I. Effect of Water Extraction on Cellulolytic Activity of DDS

Treatment	Cellulose Digested,[a] %
Control	41.3
DDS	48.3
DDS water extract	50.2
Residue from water extract	40.1

[a] Difference of 6.3 needed for significance at $P = 0.05$.

Table II. Influence of Precipitation with 95% Ethanol on Activity of DDS Water Extract

Treatment	Cellulose Digested,[a] %
Control	65.9
DDS H_2O ext.	77.1
H_2O ext. alc. ppt.	74.6

[a] Difference of 6.4 needed for significance at P = 0.05.

Table III. Effect of Acid Hydrolysis on Cellulolytic Activity of Alcohol Precipitate Fraction of DDS Water Extract

Treatment	Cellulose Digested, % Trial I[a]	Trial II[b]
Control	39.3	36.7
Alc. ppt.	54.3	...
2-hr. hydrol.	59.3	...
4-hr. hydrol.	58.6	...
6-hr. hydrol.	58.1	...
8-hr. hydrol.	58.3	...
10-hr. hydrol.	57.7	54.4
24-hr. hydrol.	...	53.4

[a] Difference (D) of 5.8 needed for significance at $P = 0.05$.
[b] $D = 5.7$.

Table IV. Cellulolytic Activity of Paper Chromatogram Fractions of a Hydrolyzate of DDS Water Extract Alcohol Precipitate

Treatment[a]	Cellulose Digested,[b] %
Control	49.0
DDS H_2O ext. alc. ppt., 24-hr. hydrol.	61.5
Paper fraction	
1	45.8
2	51.3
3	55.2
4	49.7
5	46.2

[a] Treatment additions at levels equivalent to 50 mg. of original DDS.
[b] Difference of 5.3 needed for significance at $P = 0.05$.

Table V. Proline Content of DDS and Fractions of DDS

Sample	Proline,[a] μmoles
DDS	7.4
DDS H_2O ext.	3.2
H_2O ext. alc. ppt.	2.1
Paper chromatogram fraction 3	2.1

[a] In each fraction of 50 mg. of original DDS.

Table VI. Influence of Level of Proline on Cellulose Digestion

Treatment	Cellulose Digested, % Trial I[a]	Trial II[b]
Control	29.8	26.1
Proline, μmoles		
2.5	33.7	...
5.0	34.5	...
10.0	38.7	37.4
20.0	...	36.6

[a] Difference (D) of 8.0 needed for significance at $P = 0.05$.
[b] $D = 4.0$.

Yellow color was used to detect proline. Strips were divided into five fractions. Fraction 3 was prepared to contain the proline with a minimum of other amino acids and was between R_f 0.29 and 0.35. Fraction 1 was between R_f 0 and 0.03, fraction 2 was between R_f 0.03 and 0.29, fraction 4 was between R_f 0.35 and 0.55, and fraction 5 was between R_f 0.55 and the solvent front. The sample material was removed from the fractionated paper strips by suspending the strips in distilled water for a 4- to 6-hour soaking period, followed by filtration through a fritted glass funnel. Each strip was washed with distilled water until it gave negative ninhydrin tests. The filtrate containing the amino acids from each fraction was evaporated to dryness over steam and resuspended quantitatively in distilled water. Each fraction was added to the fermentation tubes at a level equivalent to 50 mg. of original DDS.

The cellulolytic activities of the chromatogram fractions are shown in Table IV. The only fraction with a significant activity was fraction 3, which contained proline. However, it was less active than the nonchromatographed hydrolyzate.

Proline contents of the active fractions of DDS were determined and the amount was compared with the synthetic proline needed to produce a cellulolytic response comparable to that of DDS. A sample of each fraction containing 2 to 4 mg. of protein was hydrolyzed with 6.N HCl and placed on an ion exchange column for separation of the amino acids by a modification of the technique described by Hamilton (*6*).

The proline contents of DDS and its active fractions are shown in Table V. The proline content of the water extract was only approximately half that of the original DDS. Most of this proline was precipitated by alcohol. Fraction 3 from the paper chromatogram contained essentially all the proline in the alcohol precipitate.

To determine if the amount of proline in these active fractions of DDS was sufficient to explain their cellulolytic activity, the level of synthetic proline necessary for maximum cellulolytic response was determined (Table VI). No significant stimulation of in vitro cellulose digestion was obtained from additions of 2.5 and 5.0 μmoles of proline per 20 ml. of artificial rumen medium. Adding 10.0 μmoles produced a significant response which was not increased by adding 20.0 μmoles. Therefore, approximately 10 μmoles of proline per 20 ml. of artificial rumen medium seemed necessary to stimulate cellulolytic response as much as 50 mg. of DDS.

Although there may be sufficient proline in DDS to explain the cellulolytic activity of this feed, some of the fractions of DDS contained less proline than was required to obtain optimum cellulolytic activity when synthetic proline was added. The relative cellulolytic activity and the amount of proline in DDS and fractions of DDS are shown in Figure 1.

The water extract of DDS contained all the cellulolytic activity, but only 43% of the proline in DDS. There was some decrease in the cellulolytic activity in the alcohol precipitate from the water extract, along with a decrease to 28% of the original proline. There was a significant decrease in cellulolytic activity in the active chromatogram fraction of the alcohol precipitate of the water extract with no loss of proline.

Dehority *et al.* (*4*) and El-Shazly (*5*) have reported that proline can be deaminated reductively by rumen microorganisms to form valeric acid. In the current study valeric acid was found to stimulate cellulose digestion as much as

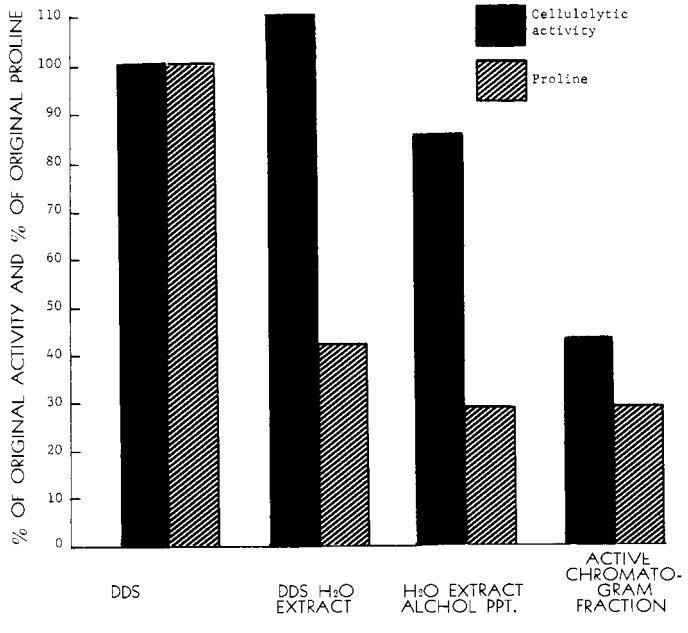

Figure 1. Comparison of cellulolytic activity and proline level in DDS and fractions of DDS

Table VII. Cellulolytic Activity of Proline and Valeric Acid after Different Periods of Fermentation

Treatment	Cellulose Digested, %			
	6 hr.[a]	12 hr.[b]	18 hr.[c]	24 hr.[d]
Control	8.9	27.6	37.2	73.9
Proline, 10 μmoles	10.3	30.8	59.1	83.2
Valeric acid, 10 μmoles	6.7	31.1	61.2	84.6

[a] Difference (D) of 7.6 needed for significance at $P = 0.05$.
[b] $D = 8.1$.
[c] $D = 7.2$.
[d] $D = 8.4$.

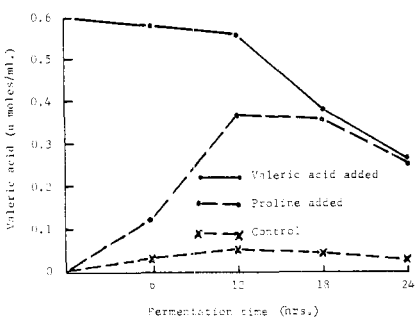

Figure 2. Valeric acid analysis of artificial rumen fluid after addition of proline or valeric acid

proline and DDS, while sugars, amino acids other than proline, B-vitamins, and several other short-chained fatty acids showed no response. However, the active fractions of DDS contained no valeric acid. The proline in DDS may have elicited its cellulolytic response through conversion to valeric acid. This was studied by adding proline or valeric acid to fermentation tubes, and analyzing the fluid for proline and valeric acid after different periods of fermentation. Cellulose digestion was also measured at these times. Free proline in deproteinized artificial rumen fluid was determined by previously mentioned procedures, and valeric acid was determined by gas chromatographic techniques.

The cellulose digestion results are shown in Table VII. No increase in cellulose digestion over that of the control was obtained by the addition of proline or valeric acid at 6 and 12 hours after the initiation of fermentation. At 18 and 24 hours' fermentation time, the addition of both proline and valeric acid resulted in significant increases in cellulose digestion over the control.

Free proline was present only in the fluid to which it had been added and only during the first 12 hours of fermentation. Valeric acid levels in the artificial rumen fluid from all treatments are shown in Figure 2. When valeric acid was added, there was an over-all decrease in valeric acid during fermentation. During the first 12 hours when the response in cellulose digestion was not significant, the valeric acid level decreased slightly. However, between 12 and 24 hours, there were a marked cellulolytic response to valeric acid and rapid disappearance of valeric acid. When proline was added, there were a sharp net decrease in free proline and a sharp net increase in valeric acid up to 12 hours, or during the time when no cellulolytic response was noted. Assuming that proline was converted to valeric acid (*4*), it appears from Figure 2 that between 12 and 18 hours the net production of valeric acid from proline stopped and utilization of that valeric acid began. Along with this, significant increases in cellulose digestion over the control were noted at 18 and 24 hours. After 18 hours the decrease in valeric acid, presumably produced from proline, was at essentially the same rate as the decrease in added valeric acid.

Discussion

The results of the initial experiments of this study verified the presence of factors in corn distiller's dried solubles (DDS) which are stimulatory to in vitro cellulose digestion by rumen microorganisms. Several chemical and physical properties associated with the active portions of DDS have been determined. Proline is the only synthetic chemical compound reported previously to be active which has these properties and consistently gives responses in cellulose digestion similar to responses to DDS. Valeric acid also gives a cellulolytic response but does not possess some of the properties of the factors in DDS.

Analyses of the active fractions of DDS revealed that valeric acid was not present; thus, it was not directly responsible for the stimulation of cellulose digestion by this fermentation feed and its various fractions. Since proline was detected in each active fraction, an attempt was made to relate it to the activity of DDS and its fractions. After quantitative partitioning of the proline from an active fraction of DDS by paper chromatography, only the fraction containing proline stimulated cellulose digestion. This indicated that proline is a source of the rumen-stimulatory properties of DDS, but failure to recover a significant portion of the activity with proline suggests that other factors in DDS may be involved. This hypothesis was supported by experiments in which the relation of activity to proline content of the various fractions of DDS was studied. If proline is the only factor in DDS which affects microbial cellulose digestion in the rumen, one should be able to predict the relative activity of a preparation by determining its proline content. Although proline was present in all the active fractions studied, its concentration was reduced considerably by fractionation without comparable loss of activity (Figure 1). Consequently, proline content alone could not be used to predict activity of the different fractions.

Valeric acid enhances the cellulolytic activity of rumen microbial cultures and has been isolated from rumen fluid (*1*). Proline has been suggested as an active compound and related to the stimulatory properties of casein and alfalfa extract (*3*). Pathways for the microbial conversion of proline to valeric acid have been suggested (*4*). Therefore, it can be hypothesized that valeric acid is a central compound in stimulating cellulose digestion by rumen microorganisms. Natural feeds which do not contain valeric acid *per se* could possibly give this stimulation through the conversion of proline to valeric acid. This suggests that the responses to proline from natural feeds and to valeric acid from some fermentation products are accomplished through similar but yet to be determined metabolic roles.

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